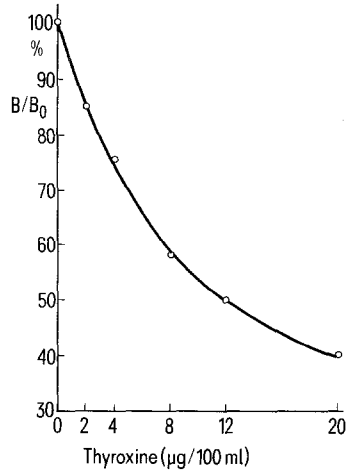


Fig. 2. Thyroxine assay. Immunoassay for thyroxine was developed using MBTM-enzyme conjugate as label and second antibody for separation of bound from unbound. To a glass tube (16×100 mm) 100 µl of a 100-fold dilution of the peak fraction of MBTM-enzyme conjugate eluted from Sephadex G-25 column was added, followed by addition of 500 µl of phosphate buffer (pH 7.0, 0.05 M) containing 0.5 mM 8-anilino-1-naphthalene-sulfonic acid-sodium salt. 50 µl of each of a series of standards containing 0, 2, 4, 8, 12 and 20 µg/100 ml of L-thyroxine in serum (Syva Corp.) were added, followed by 100 µl of a 1:400 dilution of rabbit anti-thyroxine antiserum. After incubation for 45 min at room temperature, 100 µl of 1:20 dilution of normal rabbit serum was added, followed by addition of 100 µl of goat anti-rabbit IgG. After vortex mixing, the tube was incubated for 15 min in ice water and centrifuged for 10 min at 3000 rpm. The pellet was washed twice with phosphate buffer and resuspended in 0.5 ml phosphate buffer-BSA (pH 7.5, 0.05 M, 0.1% BSA). The enzyme activity was assayed using o-nitrophenyl-β-D-galactoside as substrate. O-nitrophenol produced at the end of incubation time was measured by Gilford Stasar III spectrophotometer at 420 nm wavelength.

*Synthesis of L-thyroxine methyl ester.* L-thyroxine methyl ester was prepared by the method of Ashley and Harington[9].

*Conjugation of β-galactosidase to maleimidobenzoyl L-thyroxine methyl ester (MBTM).* 50 µl of a solution of MBTM in THF (0.2 mg/ml, 10 nmoles) was added to 1.5 ml of 0.05 M phosphate buffer (pH 7.0) containing β-galactosidase (0.5 mg, 0.93 nmole). The mixture was incubated for 2 h at room temperature. Following overnight dialysis in phosphate buffer, the mixture was chromatographed on a Sephadex G-25 column (1.5×40 cm). The fractions of eluate containing the peak of enzyme activity were used for the thyroxine assay. β-galactosidase activity was assayed by the method of Dray et al.[4] using o-nitrophenyl β-D-galactoside as substrate.

When MBTM and β-galactosidase conjugation was carried out at molar ratios of over 5 to 1, more than 97% of the enzyme was found to be conjugated with MBTM as examined by double antibody precipitation method in excess of anti-thyroxine antibody. The number of moles of MBTM conjugated per enzyme was not determined. Since it is known that β-galactosidase possesses about 10 sulfhydryl groups per molecule[11], the maximum number of MBTM attached per enzyme is 10. The practical limit of solubility of MBTM in the conjugation solvent is 10 µg/ml, restricting the molar ratio of MBTM to enzyme. The conjugation reaction was carried out immediately after dissolution of MBTM in the buffer.

Enzyme activities examined before and after the conjugation step did not show any difference, suggesting full retention of the enzyme functional groups. With the final antiserum dilution of 2400-fold, a reproducible thyroxine enzyme immunoassay was successfully demonstrated. Incubation times of the assay were 45 and 15 min respectively for competitive binding of thyroxine and MBTM-enzyme conjugate and double antibody precipitation steps (figure 2). The highest sensitivity in the assay was observed at 0–10 µg/100 ml range.

We believe that our novel approach in the use of the maleimide derivative of hapten is a convenient and efficient way for conjugating hapten to an enzyme without reduction of enzyme activity. Our model of enzyme-hapten conjugation procedure could be extended to many haptens and can improve assay sensitivity and precision.

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## A rapid micro radial electrophoretic method of protein separation on cellulose acetate membranes[1]

K.M. Rao, N. Raja and S.S. Rao

*Defence Research and Development Establishment, Gwalior-474002 (India), 13 July 1978*

*Summary.* A new rapid micromethod for protein separation under a radial electric field is described. As many as 12 rabbit serum samples could be separated in 4–6 min.

Cellulose acetate membranes are used in various methods, like double diffusion, electrophoresis and immunoelectrophoresis[2–5]. These techniques have been adopted to micro methods also[6].

In the course of investigations on micro analytical techniques applicable to biological materials of pathodiagnostic importance, we developed a simple, quick and reproducible procedure of protein separation on cellulose acetate membranes. The interesting feature of the procedure is that, probably for the first time, a circular electrophoretic separation is successfully employed for protein separation. An inexpensive apparatus was designed and fabricated in our laboratory for this purpose. It consists of a perspex sheet with mini tanks and copper strips as electrodes (figure 1).

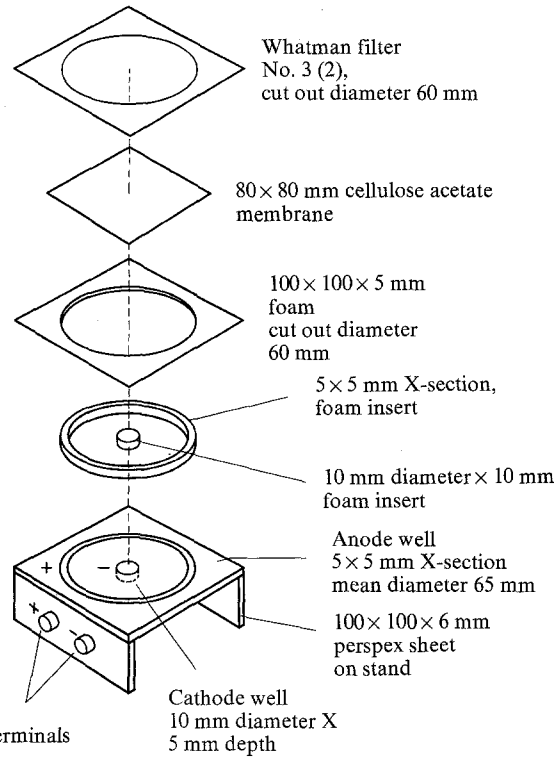


Fig. 1. Radial electrophoretic apparatus.

After making suitable impressions of pre-determined pattern for protein application over the sartorius cellulose acetate membranes 8 × 8 cm, the membranes were saturated with veronal buffer (pH 8.6, 0.075 m) and securely placed on the sponge soaked with the same buffer in the electrode compartments. Protein samples are applied about 0.6 cm from the cathode and electrophoretic separations are carried out with a constant current supply of 80 V through a power pack (Systronic electrophoresis power supply 603). Migration of the samples was observed by the bromophenol blue that is mixed with the serum samples. Adequate separation of rabbit serum proteins is achieved in about 4–6 min (figure 2). It is found necessary to change the buffer after every 2 separations to achieve satisfactory migration. Staining was usually done by Ponceau S 0.2% in 3% trichloro acetic acid for quick visualisation of the bands in 5 min. For photographic recordings and photometric scanning, they are stained again by nigrosine 0.002% in 2% acetic acid.

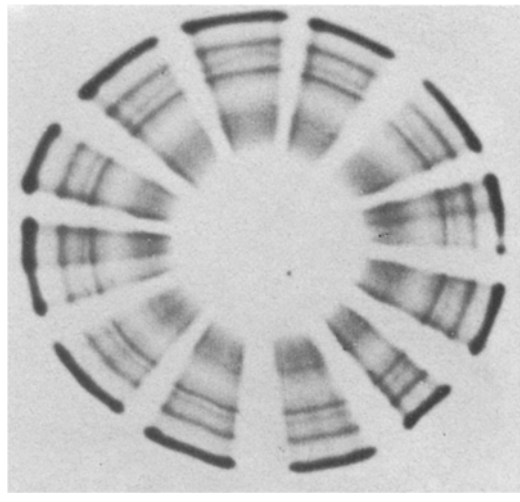

Fig. 2. Normal rabbit serum applied as 12 samples. The separation clearly shows 5 bands after 5 min of migration. The migration is from centre.

The micro radial electrophoresis is faster and requires about 15 ml of buffer for 2 separations. 12 samples can be processed simultaneously and it is very inexpensive. Preliminary work on employing this technique for immuno electrophoresis has given very satisfactory results.

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